IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:	Confirmation No.: 5804
Ferdinand Hermann BAHLMANN, *et al.*	
Serial No.: 10/522,426	Group Art Unit: 1654
Filed: March 25, 2005	Examiner: Thomas S. Heard
For: USE OF ERYTHROPOIETIN	

VIA EFS-WEB
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

THIRD DECLARATION OF PROF. DR. HERMANN HALLER UNDER 37 C.F.R. §1.132

I, Hermann Haller, hereby declare that:

1. I am the same Dr. Hermann Haller who previously submitted a first declaration in this application that was executed on May 2, 2008 ("Haller I") and a second declaration that was executed on December 16, 2008 ("Haller II"). My background and relationship to the present application are as stated in ¶¶ 1-2 of the Haller I and II declarations. I have reviewed the Office Action from the United States Patent and Trademark Office dated April 16, 2009 ("the Office Action") and I am familiar with the references cited therein as a basis for the various grounds of rejection of the claims. I am making this declaration in support of the patentability of the claims of the present application, as amended in the response filed together with this supplemental declaration.
2. At p. 9 in the Office Action the Examiner raises several objections to the Haller II declaration. First, it is alleged that the labeling of the graphs is not clear. I believe that this allegation is due, at least in part, to the fact that the original exhibits to the Haller II declaration were submitted in color. The Examiner's remark that, "there are three white bars and one black bar" (i.e., in Exhibit 8) indicates to me that the problem occurred as a result of the declaration exhibits being scanned into the U.S. Patent Office computer system in black and white, and not in color.

Provided as Attachment 1 to this declaration, therefore, is a substitute set of the Exhibits (Nos. 1-9) that is intended as a replacement for the exhibits submitted with the Haller II declaration. These 'replacement' exhibits are similar to those provided with the Haller II declaration, but there are two important differences between the replacement exhibits and the original exhibits. These differences are explained below.

3. The first difference between the replacement set of exhibits and the original Haller II exhibits is that the replacement exhibits are submitted in black and white, not in color. In place of the colored bars found in the original exhibits (e.g., Nos. 7-9) the bars of the graphs are differently shaded. In summary, the different colors have been replaced with different shadings.

4. The second difference between the replacement set of exhibits and the original Haller II exhibits concerns the legends provided for Exhibits 7, 8 and 9. Upon receipt of the Office Action, I reviewed the original exhibits and discovered a translation error in the figure legends. In particular, the 'right-most' bar in Exhibits 7-9 is identified in the original exhibits as, "non-diabetic – high-dose EPO". The word "non" is an error from the translation of the original German-language legend provided to these exhibits. The legend has thus been corrected in the replacement exhibits provided with this declaration such that it now states, "diabetic – high-dose EPO". The correction is evident from the description of the subject exhibits as set forth in ¶7 of the Haller II declaration. As stated therein, the Exhibits present a comparison among: (a) non-diabetic placebo; (b) diabetic – placebo; (c) diabetic – low-dose EPO; and (d) diabetic – high-dose EPO. The typographical correction made to the figure legend is thus entirely supported by the text of the original declaration (¶7) and no new matter is added. Furthermore, I believe that the clarification of the data presented in the declaration due to the submission of replacement exhibits 1-9 entirely overcomes the Examiner's objections to the original declaration set forth on p. 9 of the Office Action. In particular, the Examiner objected to my declaration as being unclear, incomplete and as lacking proper controls for demonstrating an unexpected result. Due to the submission of black/white forms of the bars I understand that the unclarity issue is

resolved. Due to having explained the mislabeling of the graphs I understand that also the objections against the content of the declaration are resolved. In particular, Exhibit 7 to 9 now clearly comprise, in accordance with section 7 of the original declaration, two controls, namely a non-diabetic placebo and a diabetic placebo set of experiments. The non-diabetic placebo set of experiments is a positive control indicating the process of wound healing in healthy animals. The diabetic placebo set shows as a negative control the process of wound healing in Placebo-treated diabetic animals. The dotted bar shows the process of wound healing of the present invention in contrast to an EPO treatment with conventional high doses and clearly displays the advantageous and surprising effect that using a low-dose EPO treatment in diabetic animals led to a wound healing comparable to the non-diabetic placebo treated positive control while the high-dose EPO treatment of diabetic animals showed no positive effect on the wound healing process and is comparable to the negative placebo diabetic control group. The low-dose EPO treatment led to a significantly faster healing of the wound than the high-dose EPO treatment (e.g. Exhibit 9, third and fourth bar). In view of the state of art on record and the expectations of an average skilled person such a result is totally unexpected. I request, therefore, that the Examiner withdraw his objections to my prior Haller II declaration based on the discussion above.

5. Further to the above, I understand from the Office Action that the Examiner has cited a new reference against the claims of this application, namely Published U.S. Patent Application No. US 2003/0130197 of Smith-Swintosky, et al. According to the discussion set forth at p. 3-4 of the Office Action, the Examiner considers that "ischemia" is readable on the term "wound" and thus, in his view, the invention as presently claimed is anticipated by the prior art. I respectfully disagree with the Examiner's conclusion for the reasons presented below.

6. I am informed by my European patent counsel that the claims under examination in this application are being amended by our U.S. counsel in the response that is filed to the present Office Action to refer, specifically, to diabetic wound healing, i.e., the healing of wounds caused due to, or which are otherwise attributable, to diabetes. As I explain below, the method as claimed of using low-dose EPO to

heal diabetic wounds is not taught, or even suggested, by the Smith-Swintosky et al. reference. Although Smith-Swintosky et al. does disclose the administration of low-dose EPO, its teachings concerning the treatment of ischemic rats does not disclose a wound-healing effect for the 'active' material and particularly not a healing effect for diabetic wounds such as that now recited in the pending (amended) claims under examination. Thus, for the reasons given the Smith-Swintosky reference does not teach the presently claimed method, nor would it suggest such method to one having an ordinary level of skill in the relevant field.

7. Wound healing in diabetes differs significantly, on both a molecular and a cellular level, from repair mechanisms responding to trauma in the brain, and particularly to the treatment of effects due to ischemia. That is, the mechanism of injury in the skin of diabetic patients is significantly different from the mechanisms used to treat ischemic injury to the brain. Furthermore, the tissue reaction is different in these two cases and the mechanisms of regeneration also differ considerably. In sum, diabetic wounding is dominated by hyperglycemic mechanisms, followed by glucose-induced oxidative stress with deleterious effects on stem cells and subsequent problems of fibrosis. On the other hand, brain injury due to ischemia, is dominated by endothelial cell activation and a cascade of inflammatory reactions leading to acute local inflammation. The regeneration, i.e., in the case of brain injury, is less influenced by fibrosis than is the case with regard to diabetic wounding. Therapeutic strategies in the two areas also vary considerably.

8. By me, or under my direction and control, a report has been produced which clearly sets forth the molecular and cellular differences between wound healing in diabetes and mechanisms of injury and regeneration following cerebral, i.e., ischemic, trauma. A copy of the report is provided as Attachment 2 to this declaration. From the information contained therein I believe that it is very clear that the disclosure contained in Smith-Swintosky, et al., relating to the treatment of an ischemia condition in rats, would not teach or even suggest the method of the present invention as now recited in the amended claims presented to the Examiner which, as indicated above, are directed to the treatment of diabetic wounds.

9. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements so made may jeopardize the validity of the application or any patent issuing thereon.

Date: 03-09-2009

By: _____
Hermann Haller

ATTACHMENT 1

EXHIBIT 1

Phase I

Necrosis/Skin ulceration

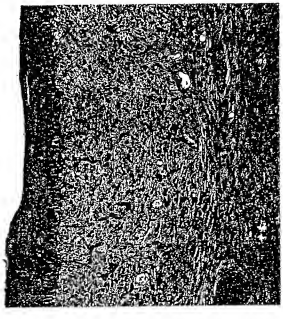
EXHIBIT 2

Phase II

Numerous granulative and resorptive Infammation with starting reepithelisation

EXHIBIT 3

Phase III



Moderate cell-rich granulative inflammation wherein reepithelisation is progressing

EXHIBIT 4

Phase IV

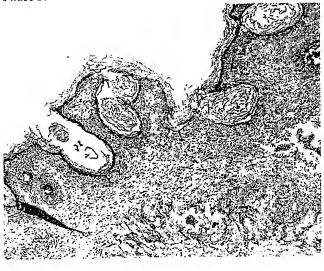


EXHIBIT 5

Phase V

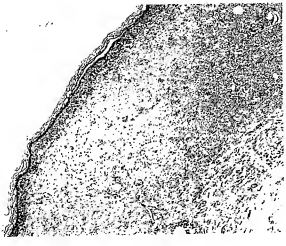


EXHIBIT 6

Phase V with vascular proliferation

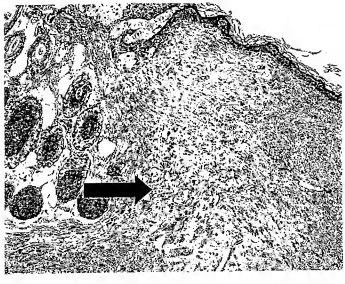


EXHIBIT 7

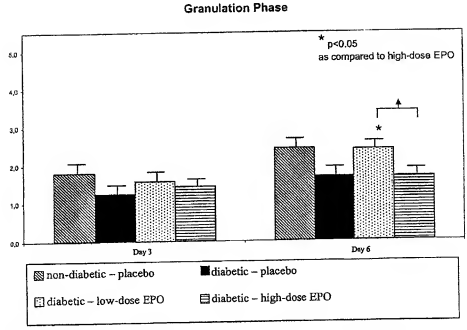


Fig. A: Process of wound healing in the different treatment groups during Granulation Phase at day 3 and 6

EXHIBIT 8

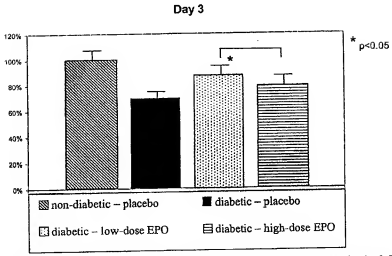


Fig. B: Relative process of wound healing in the different treatment groups at postoperative day 3. The healing process was normalised to the level of non diabetic animals.

EXHIBIT 9

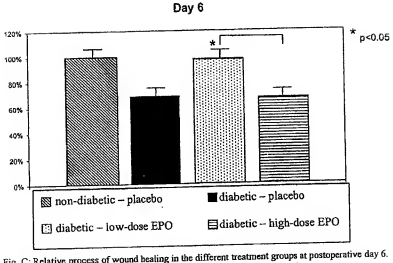


Fig. C: Relative process of wound healing in the different treatment groups at postoperative day 6. The healing process was normalised to the level of non diabetic animals.

ATTACHMENT 2

One of the questions asked was: Is wound healing in diabetes different from other repair mechanisms, especially response to trauma in the brain? - We describe in the following pages the molecular and cellular differences between wound healing in diabetes and mechanisms of injury and regeneration after cerebral trauma.

The two phenomena, although it is wound healing and regeneration in both cases are very different. Firstly, the mechanism of injury in the skin of diabetic patients and the mechanisms which take place in the brain which is mostly of ischemic nature are different. In addition, the tissue reaction is different and the mechanisms of regeneration also differ considerably. In the following two chapters we will describe first the molecular and cellular mechanisms of wound healing. Secondly, the molecular and cellular mechanisms of brain injury and repair will be described. It is obvious that diabetic wound healing is dominated by mechanisms of hyperglycemia, followed by glucose-induced oxidative stress with deleterious effects on stem cells and subsequent problems of fibrosis, while brain injury is dominated by endothelial cell activation and a cascade of inflammatory reactions leading to acute local inflammation. The regenration is less influenced by fibrosis. Therapeutic strategies in the two areas also vary considerably.

Pathophysiology of wound healing in diabetes

Wound healing in diabetes is a serious health problem world-wide. Therapies are urgently needed in order to address this serious health problem. We have identified erythropoietin (EPO) at low concentrations as a novel therapeutic agent which can be used in wound healing under diabetic conditions. EPO specifically acts on wound healing in diabetics. These findings argue that EPO has specific effects on diabetic wound healing. The pathophysiology of wound healing in diabetes is different fro wound healing in normal patients. In this article we review the known differences between diabetic wound healing and wound healing in general in order to demonstrate that our novel therapeutic approach i.e. low-dose EPO is specific for wound healing problems in diabetics.

Diabetes affects approximately 170 million people worldwide, including 20.8 million in the USA (1), and by 2030 these numbers are projected to double (2). Diabetic ulcerations, especially foot ulcers, are a leading cause of hospital admissions for people with diabetes in the developed world (3) and are a major morbidity associated with diabetes, often leading to pain, suffering, and a poor quality of life for patients. Diabetic foot ulcers (DFUs) are estimated to occur in 15% of all patients with diabetes (3) and precede 84% of all diabetes-related lower-leg amputations (4). Diabetic ulcers differ from normal wounds by impaired healing mechanisms and have a different pathophysiology. In the following pages we will outline the specificities of diabetic wound healing in order to make understandable that our novel therapeutic approach using Erythropoietin is based on these specific pathological mechanisms and are not comparable to normal wound healing. Despite the existence of protocols to standardize care, the physiological impairments that can result in a DFU complicate the healing process. Currently, the only FDA-approved growth factor and cell therapies for DFUs are not

routinely used during treatment, preventing professionals from implementing evidence-based protocols (5).

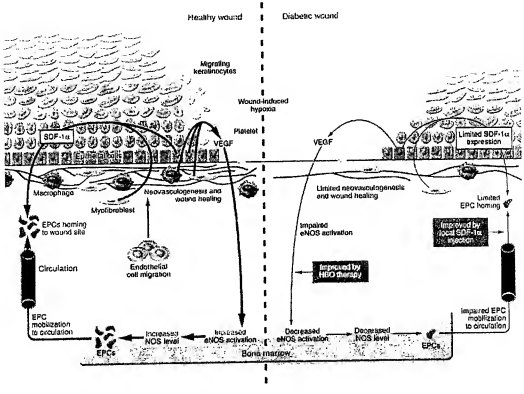
Mechanisms of wound healing in healthy people versus people with diabetes

The moment a person with diabetes suffers a break in the skin of their foot, they become at danger for amputation. Most commonly, patients have neuropathy, which could be causative. When coupled with an impaired ability to fight infection, these patients become largely unable to mount an adequate inflammatory response. Thus, the DFU that may look like a healing wound becomes a portal for infection that can lead to sepsis and require limb amputation.

Over 100 known physiologic factors contribute to wound healing deficiencies in individuals with diabetes. These include decreased or impaired growth factor production (6–8), angiogenic response (8, 9), macrophage function (10), collagen accumulation, epidermal barrier function, quantity of granulation tissue (8), keratinocyte and fibroblast migration and proliferation, number of epidermal nerves (11), bone healing, and balance between the accumulation of ECM components and their remodelling by MMPs (12). Wound healing occurs as a cellular response to injury and involves activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. Many growth factors and cytokines released by these cell types are needed to coordinate and maintain healing. In healthy individuals , the acute wound healing process is guided and maintained through integration of multiple signals (in the form of cytokines and chemokines) released by keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. During wound-induced hypoxia, VEGF released by macrophages, fibroblasts, and epithelial cells induces the phosphorylation and activation of eNOS in the bone marrow, resulting in an increase in NO levels, which triggers the mobilization of bone marrow EPCs to the circulation. The chemokine SDF-1α promotes the homing of these EPCs to the site of injury, where they participate in neovasculogenesis. Gallagher et al. (18) have shown that, in a murine model of diabetes, eNOS phosphorylation in the bone marrow is impaired, which directly limits EPC mobilization from the bone marrow into the circulation. They also show that *SDF-1* α expression is decreased in epithelial cells and myofibroblasts in the diabetic wound, which prevents EPC homing to wounds and therefore limits wound healing. The authors further show that establishing hyperoxia in wound tissue activated many NOS isoforms, increased NO levels, and enhanced EPC mobilization to the circulation. However, local administration of SDF-1 α was required to trigger homing of these cells to the wound site. These results suggest that SDF-1 α administration may be a potential therapeutic option to accelerate diabetic wound healing alone or in combination with existing clinical protocols. We have shown that treatment with EPO enhances EPC mobilization to the circulation and subsequent differentiation leading to significantly ameliorated wound healing under diabetic conditions.

Molecular analyses of biopsies from the epidermis of patients have identified pathogenic markers that correlate with delayed wound healing. These include overexpression of c-myc and nuclear localization of α -catenin (13). Coupled with a reduction in and abnormal localization of EGFR and activation of the glucocorticoid pathway, keratinocyte migration is inhibited (13, 14). At the nonhealing edge (callus) of DFUs,

keratinocytes show an absence of migration, hyperproliferation, and incomplete differentiation (13, 14). Fibroblasts demonstrate a phenotypic change as well as decreased migration and proliferation. In contrast, cells from an adjacent nonulcerated area display the appearance of a normal phenotype but are still physiologically impaired. However, they are able to respond to administration of additional growth factors or cellular therapy. Microarray analyses of patient biopsies have confirmed these clinical findings by showing that the transcription profiles of epithelial cells from the two locations (callus and adjacent nonulcerated skin) are distinct and recognizable (14).



(adapted from Brem et al. *J. Clin. Invest.* 117:5)

Bone marrow progenitors in diabetic wound healing

Bone marrow–derived endothelial progenitor cells (EPCs) play a significant role in the process of neovascularization in response to ischemic conditions, as may be the case in diabetic wounds complicated by decreased peripheral blood flow. Gallagher and colleagues (18) have shown that EPCs in the bone marrow respond to ischemia by following chemokine gradients, which results in the homing of these cells to sites of hypoxia, where they then participate in the formation of new blood vessels. Bone marrow–derived EPCs are mobilized by eNOS activation in the bone marrow, a process that the authors hypothesized is impaired in

diabetics, thus preventing these cells from reaching the wound site in significant numbers. Hyperoxia has been shown to stimulate eNOS activation in some tissues (19). Therefore, to test the effect of hyperoxia on eNOS activation and EPC recruitment, the authors wounded and later exposed diabetic and nondiabetic mice to hyperbaric oxygen therapy (HBO). Results showed that although the total numbers of active EPCs were much lower in diabetic mice than in controls, hyperoxia does indeed spur the mobilization of EPCs from the bone marrow to the bloodstream. EPC mobilization into the bloodstream occurs through an increase in bone marrow eNOS activation as a result of hyperoxia. The increased eNOS stimulates NO production, which in turn helps to produce EPCs from the bone marrow. Though hyperoxia can increase the levels of circulating EPCs in the bloodstream, the cells are not effectively mobilized to the wound site, creating another roadblock in the path to healing.

The term "homing" relates to the signals that attract and stimulate the cells involved in healing to migrate to sites of injury and aid in repair. EPC recruitment to the wound site depends on ischemia-induced upregulation of stromal cell–derived factor–1 α (SDF-1 α). Gallagher et al. (18) also report a decrease in *SDF-1* α expression particularly by epithelial cells and myofibroblasts derived from wounds of streptozocin-induced diabetic mice. The decrease in SDF-1 α was found to be responsible for decreased EPC homing. This defect was largely corrected by the simultaneous administration of HBO and SDF-1 α at the wound site. These results imply that the decreased expression of *SDF-1* α by epithelial cells and myofibroblasts may be responsible for the lack of EPC homing to the periphery of diabetic wounds. This work is extremely important because it underscores the complexity of regulatory responses in diabetic wounds and explains the inconsistent response to currently approved hyperoxia protocols (i.e., HBO) in patients with diabetes (20).

These findings correlate with our observations using EPO as a novel therapeutic strategy. We have previously shown that PO at low concentrations induce release of progenitor cells from the bone marrow and leads to an increase in differentiated progenitor cells in the blood of diabetic patients.

Low-dose EPO therapy - From bench to bedside

A combination of therapeutic approaches is likely to lead to a successful treatment outcome for diabetic wounds. However, EPCs seem to be ideal candidates for in vivo cell-based therapies correcting the EPC deficit inherent to diabetic wounds. Our novel strategy using PO directly addresses this specific deficit in diabetic patients.

Clinically, approved evidence-based protocols based on adequate off-loading (21) coupled with the use of FDA-approved biological therapies that have undergone rigorous controlled randomized trials (5, 22) must be utilized. These FDA-approved treatments are clinically efficacious and include PDGF-BB (23), fibroblasts delivered in an absorbable mesh (24), and fibroblasts and keratinocytes delivered in type 1 collagen (25). Potential treatments targeting eNOS activation and EPC recruitment such as low-dose EPO therapy might further stimulate healing. Wound progression should be monitored with a Wound Electronic Medical Record. This allows non-healing wounds (those that have not healed after 2 weeks of treatment) to be objectively measured and treatment to be tailored accordingly. These studies using EPO are under way.

From the laboratory perspective, there is compelling evidence that PO directly affects the specific defects in wound healing of people with diabetes as well as to provide understanding of the molecular and cellular etiologies of patients with foot ulcers. This powerful new technology could potentially be applied to people with diabetic wounds in the near future. One of the major remaining steps is the integration of our approach into a coordinated effort to make the technology developed at the bench available to patients at the bedside. It is therapies like our approach, starting at the molecular and cellular level that will help to eliminate amputations in patients with diabetes.

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Pathophysiology of injury and regeneration in the brain

Stroke is a complex condition arising from interplay of factors, such as genetic predisposition and others, that impinge on the integrity of blood vessels, interfering with the blood supply to the central nervous system (CNS) ((2) (3). The risk factors for stroke include high blood cholesterol, hypertension, low physical activity, hyperhomocysteinemia (resulting from disturbed methionine metabolism) and smoking ((4) (5) (6)). Basically two types of stroke have been demonstrated in humans: (1) that induced by a total loss of blood flow to the brain, such as during a cardiac arrest, (2) cerebral ischaemia arising from a focal loss of blood flow to the brain due to arterial blockage ((7))

Ischemic injury to neurons is mainly caused by the interruption of blood flow, hypoxia, ATP depletion and subsequent re-oxygenation of the brain in ischaemia-reperfusion ((8)). It has been observed that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are predominantly involved in the pathogenesis of stroke. They also play an important role in the exacerbation of the phase following stroke by triggering off pro-apoptotic pathways that reduce the survival chances of the neu)rons ((9) (10); (11).

In cerebral ischemia there is an ischaemic gradient that can be divided into a core, which is the central ischaemic zone, and the penumbra, which is located in more peripheral zones. The core of the cerebral infarct is not recoverable but the penumbra may recover and is identified as a target for the development of therapeutic strategies. In the penumbra, functional alterations occur in the neurons and glial cells. The principal pathological processes in acute CNS injury (like stroke, mechanical trauma, or subarachnoid haemorrhage) involve pathological permeability of the blood brain barrier (BBB), energy failure, loss of cell ion homeostasis, acidosis, increased intracellular calcium, excitotoxicity and free radical-mediated toxicity. This can lead to ischaemic necrosis or apoptosis with associated loss of calcium and glutamate homeostasis ((3); (12)). Experimental models of stroke have been developed in animals in an attempt to mimic the events of human cerebral ischaemia. The focal model involves the transient or permanent occlusion of the middle cerebral artery (MCA) to be used as a model of cerebral ischaemia ((13)). A global model has also been developed to mimic human cardiac arrest and involves the bilateral occlusion of the carotid and vertebral arteries leading to the development of stroke ((7)). Cellular models have provided useful tools for the study of ROS-mediated mechanisms of cellular dysfunction ((14), b (15).

In order to preserve the integrity of cells from oxidative damage, cells have evolved different mechanisms to scavenge various potentially damaging species.

These antioxidant defences include radical scavengers like α -tocopherol (vitamin E), α -carotene and ascorbate (vitamin C) and enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPx) ((14), (15)). Ascorbate is the most effective plasma antioxidant and can prevent lipid peroxide formation resulting from leucocyte activation ((2)). Ascorbic acid (AA) cannot penetrate the BBB but the oxidized form of ascorbic acid, dehydroascorbic acid (DHA), can enter the brain by facilitative transport ((16)). The non-enzymatic antioxidant mechanism involves reduced glutathione (GSH), which is a tripeptide (α-glutamylcysteinylglycine) that can donate electrons to oxidized species by virtue of its sulfhydryl moiety. NADPH is the source of reducing equivalents needed to replenish GSH stores ((17).

Oxidative stress causes damage in both acute and chronic neurodegenerative diseases like Parkinson disease (PD), amyotrophic lateral sclerosis (ALS) and stroke ((18), (19)). Antioxidant enzymes like glutathione peroxidase (GPx), catalase and SOD have proved useful pharmacological agents in models of neurodegeneration. Recently, SOD mimetics, are being explored as a possible treatment option and have emerged in antioxidant therapeutics ((17)). Three isoforms of SOD, the dimeric copper/zinc SOD (CuZnSOD, SOD1), manganese SOD (MnSOD, SOD2) and tetrameric, proteoglycan-bound Cu/Zn extra cellular SOD (ecSOD) are found, each with a specific distribution. SOD1 is cytosolic and nuclear; SOD2 is a mitochondrial enzyme while ecSOD is localized in cerebrospinal fluid, cerebral vessels and extracellular space ((19) (20)).

Early events during cerebral ischaemia include phospholipid metabolism, release of free fatty acids from phospholipids, production of lipid peroxides and ROS from polyunsaturated fatty acids, and increased release of excitatory amino acids (EAA) like glutamate. These also happen to be major factors implicated in neuronal injury in stroke and other neurodegenerative diseases ((17); (26)). One feature of stroke is that hypoxia causes increase in intracellular calcium ($[Ca^{2+}]_i$) in almost all cells. These changes stem from the activation of various plasma membrane calcium conductances including voltage operated calcium channels and ligand-operated channels ((27)).

Glutamate is the major EAA in the brain and acts through its ionotropic receptors. Ischaemia causes glutamate accumulation in the interstitial space because of enhanced efflux of glutamate and reduction of glutamate uptake. Hypoxia leads to depletion of ATP reserves and this causes an uncontrolled leakage of ions across the plasma membrane, leading to membrane depolarisation and neurotransmitter release, for example glutamate and dopamine. The excessive glutamate can lead to brain damage through membrane depolarization, with subsequent calcium influx via glutamate receptor operated ion channels ((3); (24); (27). The neurotoxic effects of glutamate can be potentiated by the presence of free radicals generated by a mixture of xanthine and xanthine oxidase, a well-recognised superoxide and hydroxylradical generating system ((29)). Glutamate interaction with its receptors causes phospholipase activation (there is considerable evidence implicating phospholipase A_2 (PLA_2) activation in transient ischaemia and contributing to neuronal

damage) ((36). Activated phospholipases act on the phospholipids in the cell membrane and release arachidonic acid, which undergoes further metabolism by cyclooxygenase/lipoxygenase (COX/LOP) to produce arachidonic acid metabolites together with ROS; the end result of this chain of events is cell death ((26) (31)). Quinolinic acid is an endogenous metabolite of tryptophan and is a selective agonist at n-methyl-d-aspartate (NMDA) receptors ((32)). Homocysteine may have an excitotoxic effect on different NMDA receptors subtypes and may increase hydroxyl radical formation ((4)). Ishige et al in an experimental stroke model system using oxidative stress (mouse hippocampal cell line HT-22) showed that the loss in cellular GSH up to 85% of the control level caused a 5 to 10-fold increase in levels of ROS. However, a greater GSH loss was found to stimulate the mitochondria to produce a 100-fold increase in ROS ((28)). Another free fatty acid, docosahexaenoic acid (DHA) is also an important source of ROS and lipid peroxidation ((31). Membrane lipids are prone to oxidative damage because they not only have a high percentage of polyunsaturated fatty acid but also because of the localisation of systems producing ROS in the membrane ((33)).

It has become increasingly evident that ROS play a significant role in reoxygenation injury. During hypoxia and reperfusion (H-R) vascular endothelium is a primary site of ROS generation that can lead to cell death. Since oxidative stress is known to induce loss of mitochondrial membrane potential dilated cardiomyopathy, in addition to cytochrome c release from the mitochondria ((34)).

The reperfusion that follows cerebral ischaemia uncouples oxidative phosphorylation and increases ROS generation and lipid peroxidation ((17)). Reperfusion after ischaemia causes reoxygenation and provides an excessive substrate supply for oxidation reactions, hence causing excessive production of ROS in mitochondria and this can lead to depletion of endogenous antioxidants ((19); (35)). The reperfusion period required for cerebrovascular ROS generation is much shorter than that required for brain damage and oedema formation. Therefore, it has been suggested that oxidative injury plays a role in cerebrovascular damage after ischaemia reperfusion, thus predisposing the brain tissue to damage ((36)). Oxidative stress caused by ROS may either be through effects on proteins, lipids and DNA or intracellular signaling pathways that involve changes in regulation of gene expression (; (23) (45); (46)). So, ROS and RNS can either alter the redox state of cells to affect specific pathways or oxidatively modify proteins ((47)).

Cerebral ischaemia activates different pathways like signaling mechanisms, gene transcription and enzyme formation by instituting changes in the redox state of cells. Matrix metalloproteinases (MMPs) and serine proteases, so induced and activated, attack the integrity of the BBB by the breakdown of the extracellular matrix around cerebral blood vessels and neurons. ROS and RNS can regulate the redox state in the cells and hence affect signaling pathways that transcribe, induce and activate these enzymes. Important MMPs found in the brain include gelatinases, stromelysins, and membrane-type metalloproteinases. These MMPs can exacerbate the phase following an acute stroke. Previous studies have shown ((48)) that the activation of MMPs is mediated by the S-nitrosylation by NO, an ischaemic attack with subsequent reperfusion induced

MMP-9 in brain cells. Vesicles containing preformed inactive MMP-9 are seen in neutrophils and can be released during the inflammatory process. Paradoxically, high nitric oxide (NO) concentration was also found to inhibit MMP-9 activation at the endothelial cell/tumour cell interface ((48)).

Damage induced by ROS can trigger cell death by influencing pathways that reduce the survival potential of cells. It has been suggested ((23)) NO and $O_2^{\bullet}$ may contribute to damage of nuclear genetic material through the formation of peroxynitrite. Low levels of these species trigger apoptosis but higher levels cause the cell to undergo necrosis. Possible mechanisms by which the apoptotic effects are mediated could involve mitochondria, DNA repair enzymes and death membrane receptors. A large body of evidence has collected regarding the role of mitochondrial apoptotic pathway after ischaemia ((49)).

ROS has been shown to affect genetic expression. NF- α B affects genes encoding pro-inflammatory cytokines, adhesion molecules, anti-oxidant enzymes and growth factors ((9)). Activation of NF- α B by H_2O_2 or hydroperoxides in neurons has been shown to have anti-apoptotic effects and is protective against glutamate exposure, glucose deprivation, hypoxia and low K^+. Conversely, in microglial cells and astrocytes, activation of the same transcription factor leads to production of neurotoxic oxyradicals and excitotoxins as well as increase in nitric oxide synthase (NOS) and NO production, promoting neuronal cell death after ischaemia. After cerebral ischaemia, both NF- α B and Akt have been observed to undergo activation ((3); (9); (55)). It is also known that Akt is involved in the activation of NF- α B, which may be involved in inducing apoptosis ((9); (47)). We conclude that the reactive species (ROS and RNS) along with inflammation are important players in the neuronal injury associated with stroke. The free radicals themselves or the molecules that they influence or interact with, as well as the intracellular signaling pathways leading to the development of apoptosis or necrosis in a temporal way following H-R represents potential therapeutic targets for the treatment of neuronal damage associated with stroke. Thus, the prevention of stroke should logically involve the augmentation of natural antioxidant reserves of the brain, and the therapeutic agents can be the molecules mimicking natural radical scavengers of the body.

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